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(54) Title: PROCESS FOR SEPARATING ENANTIOMERIC SUBSTANCES, AND A SEPARATING MEDIUM FOR CARRYING OUT THE PROCESS						
(57) Abstract						
<p>A process for separating enantiomeric substances with the aid of a separating medium is characterised in that the substances are contacted with the separating medium, which consists of at least a cellulase enzyme, a derivative or a fragment thereof and which, optionally, is immobilised on a carrier, whereupon the substances are eluted separately. A separating medium for separating enantiomeric substances is also described.</p>						

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PROCESS FOR SEPARATING ENANTIOMERIC SUBSTANCES, AND A
SEPARATING MEDIUM FOR CARRYING OUT THE PROCESS

The present invention concerns a process for separating enantiomeric substances, as well as a separating medium for carrying out the process.

The molecules of most substances with biological activity, e.g. medicines, herbicides, pheromones and insecticides, lack a centre or plane of symmetry, which makes these substances optically active. A substance which has such a spatial configuration that it cannot be covered by its own mirror image is called a chiral substance. The two mirror images of a compound, also called enantiomers, often occur in a racemic mixture, where the two enantiomers are present in an amount of 50% each. The enantiomers of, for instance, a medicine often are of different therapeutical value, since they exhibit differences in pharmacokinetics, pharmacodynamics and toxicology. In many cases, only one enantiomer in a racemic mixture possesses the desired activity, while the other may be devoid of effect or give rise to side effects of varying degrees of seriousness. Mention may here be made of the disastrous side effects of the medicine neurosedyn. To achieve optimum therapeutical effect with a minimum of unwanted side effects, a separate enantiomer or non-racemic mixtures are often administered.

From the environmental point of view, it is further of great importance that herbicides and other types of biocides are used with an optimum composition of enantiomers, and that effective methods of analysis are available to enable control of the transport and transformation of the individual enantiomers in different ecosystems.

In processes currently used for separating optical isomers, chromatography with a chiral stationary phase is often used. The purity of enantiomers can be chromatographically determined in starting material, end products and various pharmaceutical preparations. Further, chroma-

tographic methods are effective e.g. for quantifying enantiomers in biological liquids and, consequently, for characterising the effects of individual enantiomers in biological systems. Chromatography may also be used for isolating adequate amounts of enantiomerically pure substances which cannot be produced by stereoselective synthesis, or which require expensive starting materials. In addition, chromatography may be used for isolating enantiomeric metabolites from complicated biological samples, e.g. of urine and tissue.

Columns with different types of chiral, stationary phase are at present used in this field. However, such columns are, as a rule, much too specific, and are only capable of separating a narrow spectrum of compounds. The different packing materials are often difficult to make, as well as expensive, and columns used for preparatory purposes are usually uneconomic.

Thus, there is a great need in many fields for a reliable, expedient and inexpensive process for separating enantiomeric substances, in particular enantiomers in racemic mixtures. Further, there is urgent need of a separating medium which is easy and inexpensive to make, and has a long service life.

Swedish printed application 8303221-9 (Hermansson) discloses separation of different substances, preferably optical isomers, with the aid of the transport protein orosomucoid which is immobilised on a carrier in a column, e.g. silica gel particles. This publication further describes various prior art techniques for separating enantiomers, and states that the disadvantages of these known techniques can be obviated by separation with the aid of orosomucoid immobilised in a separation column.

The object of the present invention is to obviate these inconveniences by providing a process for separating enantiomeric substances, and a separating medium for carrying out the process.

This object is achieved by a process and a separating medium of the type mentioned in the introduction to this specification, said process and said medium having the distinctive features recited in the appended claims. In a 5 preferred embodiment of the invention, optical isomers, e.g. enantiomers, are chromatographically separated by being contacted with the stationary phase which consists of cellulase, a derivative or a fragment thereof immobilised on a carrier in a separation column, whereupon the 10 optical isomers are eluted separately.

Compared with the prior art, the present invention has several advantages. Thus, the starting material and the separating medium, i.e. the cellulase, the derivative or the fragment thereof, are easy and inexpensive to make, 15 and pure also in large quantities. Further, the cellulase, the derivative or the fragment thereof can be effectively immobilised on a carrier surface, e.g. of silica gel, as is the case in a preferred embodiment of the invention. In addition, the column packed with the separating medium 20 can, in a chromatographic method of analysis, be used for separating extremely hydrophobic enantiomers with the aid of simple mobile phases. The inventive separating medium further has high stereoselectivity and a capacity for several substances, and is thus suitable for preparatory 25 separations of enantiomers. There are also several possibilities of controlling the retention times through the composition of the mobile phase. Finally, the stationary phase containing the cellulase, the derivative or the fragment thereof has excellent stability, and is thus 30 suitable for routine analyses.

The preferred embodiment of the present invention differs from the invention described in, for instance, the above-mentioned Swedish printed application 8303221-9 (Hermansson) mainly in that the separating medium of the 35 invention is based on cellulase which, unlike orosomucoid, is not a transport protein. There are considerable diffe-

rences as to structure and function between these two proteins.

Further, the cellulase, the derivative or the fragment thereof often has a high capacity as separating
5 medium in chromatographic methods according to the invention.

For instance, enantiomers were separated in a sample
of 30 µg of the β blocking drug propranolol in an injection volume of 500 µl in analytical column, and the same
10 amount of propranolol could also be separated in an injection volume as small as 20 µl. Such a high capacity greatly facilitates the quantification of different enantiomers.

The prior art does not embrace using the enzyme cellulase, a derivative or a fragment thereof, for separating optically active isomers. According to the invention, cellulase has proved to be highly suitable for use in this area, mostly because of its spatial configuration with a multiplicity of accessible surface groups and
20 active sites.

In nature, enormous amounts of cellulose are broken down every year, mainly by fungi and bacteria. The first steps in the breaking-down process of cellulose are catalysed by cellulase enzymes which hydrolyse β -1,4-bonds in
25 the cellulose chains. Owing to its high crystallinity and frequent association with other substances, e.g. lignin, the cellulose is extremely difficult to hydrolyse, and the cellulases have therefore developed towards a high degree of specialisation. Usually, the cellulases are subdivided
30 into endoglucanases capable of hydrolysing internal bonds in the cellulose chain, and exoglucanases or cellobiohydrolases attacking from the nonreducing end and generally giving the product cellobiose. A very ambitious effort to classify cellulases according to homology in the
35 amino acid sequence has been made by Henrissat et al (see Cellulase Families Revealed by Hydrophobic Cluster Analysis. Gene, 81 (1989) 83).

The mould fungus *Trichoderma reesei* is an effective producer of cellulases, and some mutants yield more than 20 g enzyme per litre of culture liquid, of which about 50% is made up of cellobiohydrolase I (CBH I). *T. reesei*

5 mainly forms four different cellulases, i.e. two endo-glucanases and two cellobiohydrolases (CBH I and CBH II). The enzymes, as well as the corresponding genes, are well characterised (see J. Knowles et al, Cellulase families and their genes. *Tibtech*, 5 (1987) 255). The molecular

10 weights of cellulases are in the range of 50-65 kD. Thus, they belong to the category of large monomers, and all of them contain carbohydrates. Further, they are isoelectric at low pH values (3.5-6.0). The cellulases produced by *Trichoderma* all have a common structure, the main part of

15 the enzyme being a catalytic domain to which an additional bonding domain consisting of about 30 amino acids is connected via a flexible link. The bonding domain has a characteristic amino acid sequence and a three-dimensional structure (see P. Kraulis et al., Determination of the

20 three-dimensional structure of the C-terminal domain of cellobiohydrolase II from *Trichoderma reesei*. A study using nuclear magnetic resonance and hybrid distance geometry-dynamical stimulated annealing. *Biochemistry*, 28 (1989) 7241). In the case of CBH II, also the three-

25 dimensional structure of the catalytic domain is known (T. Bergfors et al., Crystallization of the core protein of cellobiohydrolase II from *Trichoderma reesei*. *J. Mol. Biol.* 209 (1989) 167). The majority of the carbohydrates of the enzymes are to be found in the flexible link, where

30 they are bonded via O-glycoside bonds to serine or threonine residues. The bonding domain increases the catalytic activity of the enzymes towards crystalline cellulose, probably by achieving a high and effective cellulase concentration on the fibre surface proper.

35 Owing to their structure, the cellulases are especially useful in the process according to the invention. Thus, the cellulase molecule senses the slight structural

difference between e.g. two enantiomers, and bonds one enantiomer more strongly than the other, which may be shown in a chromatogram after elution with a suitably eluant. The phrase "a cellulase, a derivative or a fragment thereof", as used throughout the present specification and claims, is meant to include all natural cellulases, cellulase derivatives and cellulase fragments. It will be obvious to anyone skilled in the art that the cellulase molecule can be modified as to the amino acid sequence, e.g. by genetic engineering, without substantially changing its properties as separating medium according to the invention, for which reason the phrase "a cellulase, a derivative or a fragment thereof" is meant to include also such modified cellulases. A preferred embodiment of the invention uses at least one of the cellulases cellobiohydrolase I (CBH I), cellobiohydrolase II (CBH II), endoglucanase I (EG I) and endoglucanase III (EG III), or a derivative thereof.

In a preparation process, CBH I and CBH II were obtained from a culture filtrate of the fungus *Trichoderma reesei* QM 9414 by affinity chromatography, p-amino-benzyl-1-thio- β -D-cellobiocide being used as ligand and connected to Sepharose 4B. Then, elution with 100 mM lactose was carried out (for CBH I), or 10 μ M cellobiose (for CBH II), whereupon these cellulases were removed by diafiltrating on membranes of the type PM-10 (Amicon, USA). The resulting mixtures of cellulase isoenzymes were further fractionated by ion exchange chromatography. The homogeneity was tested both with SDS-PAGE and IEF-PAGE. Components having a pI value of 3.9 (CBH I) and 5.9 (CBH II) were used in most of the tests, but the results with the isoenzyme mixtures were substantially identical. The molar adsorption coefficients are $73,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for CBH I, and $75,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for CBH II, and are unchanged for the raw proteins.

In a preferred embodiment of the invention, cellulase, a derivative or a fragment thereof is immobilised on a carrier in a column used for chromatography, the cellulase, the derivative or the fragment thereof forming the stationary phase together with the carrier. The chromatographic separation column used in the inventive process may be any prior art column which can be packed with the separating medium and the carrier. The mobile phase may be any suitable mobile phase. In another preferred embodiment of the invention, the mobile phase may be a mixture of chiral cellulase, a derivative or a fragment thereof, in a liquid chromatographic separation process, the stationary phase being non-chiral.

The carrier for immobilising the separating medium may be any suitable carrier but is, in a preferred embodiment of the invention, silica gel or a derivative thereof, such as diol silica gel. When the cellulase is to be immobilised in the stationary phase of the chromatographic column, periodic acid (H_5IO_6) is, according to one embodiment of the invention, initially added to an aqueous suspension of diol silica gel produced in accordance with prior art methods. The resulting aldehyde silica gel is then carefully washed with demineralised water which has been cleaned in a filtering system of the type Milli-Q. Then, the aldehyde silica gel is transferred to a phosphate buffer solution (pH 7) of the cellulase. Sodium cyanoboron hydride ($NaBH_3CN$) is also added to the solution. The vessel containing the reaction mixture is left for two days on a tilting table, whereupon the material is washed with a phosphate buffer. Finally, the material is packed by so-called slurry technique in steel columns intended for chromatographic purposes.

In the reaction, the aldehyde group reacts with the amino group resulting in an imine, i.e. a Schiff's base. The imino group is reduced by the sodium cyanoboron hydride to an amino group which serves as a bond between

the silica gel and the cellulase. Thus, the cellulase is immobilised covalently on the silica gel derivative.

The separating medium according to the invention, i.e. the cellulase, the derivative or the fragment thereof, may be used for separating optically active isomers with the aid of chromatographic and kindred techniques, e.g. electrophoresis and liquid-liquid extractions (so-called two-phase system) for separating, determining and isolating enantiomeric substances, but above all optically active substances, e.g. enantiomers in racemic mixtures.

Preferred examples of optically active substances to which the inventive process and separating medium are applicable include some medicines, e.g. β blockers, herbicides, pheromones and insecticides. However, it will be obvious to anyone skilled in the art that other compounds may also be separated. Thus, the invention is a necessary and important complement to existing processes for separating optically active compounds, but it may also more or less replace existing processes.

The invention will now be described in more detail, reference being had to the accompanying drawings, in which

Figs 1-3 represent chromatograms illustrating the separation of the enantiomers of three different, optically active pharmaceutical substances,

Fig. 4 represents chromatograms which illustrate the reproducibility of stationary phases based on the separating medium of the invention, and

Fig. 5 illustrates the stability of the inventive separating medium, both as to retention and as to effectiveness.

In tests, stationary phases with immobilised cellulase have shown excellent separation capacity (high stereoselectivity and high separation effectiveness) for a large number of enantiomeric substances, e.g. several β blockers. Inter alia the three pharmaceutical substances propranolol (see Fig. 1), alprenolol (see Fig. 2) and prilocaine (see Fig. 3) have been tested. The two first-men-

tioned substances are β blockers, whereas the last-mentioned substance is a local anaesthetic. It has also been possible to separate the gastric-acidity-inhibiting drug omeprazole, which however is not shown here. The separations of Figs 1 and 2 were carried out with the cellulase silica gel phase packed in a steel column. The mobile phase was an acetate buffer having a pH of 4.7 and containing 0.5% 2-propanol. The mobile phase for the separation of prilocaine also contained a quaternary ammonium compound in the form of 1.1 mM tetrabutyl ammonium. The present invention results in extremely high stereoselectivities and complete dissolution of the enantiomers with the use of uncomplicated, water-base and mobile phases, as is apparent from the chromatograms shown. The α value is defined as the ratio between the retention times for two enantiomers, and as high α values as 1.5-7.2 were obtained. The retention times for enantiomers, e.g. of the drugs in Figs 1-3 and the drug metoprolol, can be controlled with the aid of the pH value (see Table I below). The addition of an anionic component (see Table II below), such as octane sulphonate, to the mobile phase also enables control of the retention time, in this case for the drug labetalol.

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TABLE I
Control of the retention time with the aid of pH

Sample	pH		
	4.7	6.8	
	k'_1	α	R_s
Propranolol	0.46	2.6	4.6
Alprenolol	0.16	5.1	4.8
Metoprolol	0.10	1.7	0.7
Prilocaine	<0.01	-	-

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k'_1 is the capacity factor for the enantiomer first eluted; $k' = (t_R - t_0)/t_0$, wherein t_R is the retention time for the sample, and t_0 is the time it takes for an unretarded substance to pass the column.

5 $\alpha = k'$ for the enantiomer last eluted / k' for the enantiomer first eluted.

R_s is the chromatographic dissolution of sample peaks, completely separated substances having a value of 1.5.

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TABLE II
Control of the retention time (k') by adding an anion
(octane sulphonate)

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Sample	Octane sulphonate (mM)			
	0	5		
	k'_1	α	k'_1	α
(RR/SS)-	0.25	1.92	0.52	1.52
labetolol				

As is apparent from Table 1, the capacity factor, which is the measure of the retardation of the substance, 25 for such a comparatively hydrophobic substance as propranolol can be reduced to a k' value of 1 or below by reducing pH from 6.8 to 4.7, and still be separated with a high stereoselectivity (α). The retention time for hydrophilic ionised amines, e.g. labetolol, can be increased by adding 30 an ion-pair-forming agent to the mobile phase (see Table II).

As is apparent from the chromatograms in Fig. 4, stationary phases based on immobilised cellulase can be produced with good reproducibility. A good correspondence in 35 the separation of an amino alcohol was obtained with two different batches of immobilised cellulase silica. Stationary phases based on cellulase silica also have excel-

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lent stability, as is apparent from Fig. 5. The retention for the enantiomers and the effectiveness of the separation column was on the whole constant, even after continuous operation during one month with a buffer as mobile
5 phase. During this period, several different samples were run through the column, and many different types of mobile phases were used.

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CLAIMS

1. A process for separating enantiomers with the aid
5 of a separating medium, characterised in
that the enantiomers are contacted with said separating
medium which is at least a cellulase enzyme, a derivative
or a fragment thereof and which, optionally, is immobi-
lised on a carrier, whereupon the enantiomers are eluted
10 separately.

2. The process of claim 1, characterised in
that the enantiomers to be separated are selected
amongst (D,L)-propranolol, (D,L)-alprenolol, prilocaine
and omeprazole.

15 3. The process of any one of the preceding claims,
characterised in that the cellulase enzyme,
the derivative or the fragment thereof is immobilised on a
carrier of silica gel or a derivative thereof.

4. The process of any one of the preceding claims,
20 characterised in that the cellulase enzyme,
the derivative or the fragment thereof constitutes,
together with the carrier, the stationary phase in a
column used in a chromatographic method.

5. The process of claims 1 and 2, characterised in
25 that the cellulase enzyme, the derivative
or the fragment thereof is present in free state in
the mobile phase in a column used in a chromatographic
method.

6. The process of any one of the preceding claims,
30 characterised in that the cellulase enzyme is
at least one of cellobiohydrolase I, cellobiohydrolase II,
endoglucanase I and endoglucanase III, or a derivative or
fragment thereof.

7. The use of a separating medium for separating
35 enantiomers, characterised in that the sepa-
rating medium is at least a cellulase enzyme, a deriva-

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tive or a fragment thereof and which, optionally, is immobilised on a carrier.

8. The use of claim 7, characterised in that the cellulase enzyme, the derivative or the fragment
5 thereof is immobilised on a carrier of silica gel or a derivative thereof.

9. The use of claims 7 and 8, characterised in that the cellulase enzyme is at least one of cellobiohydrolase I, cellobiohydrolase II, endoglucanase I
10 and endoglucanase III, or a derivative or fragment thereof.

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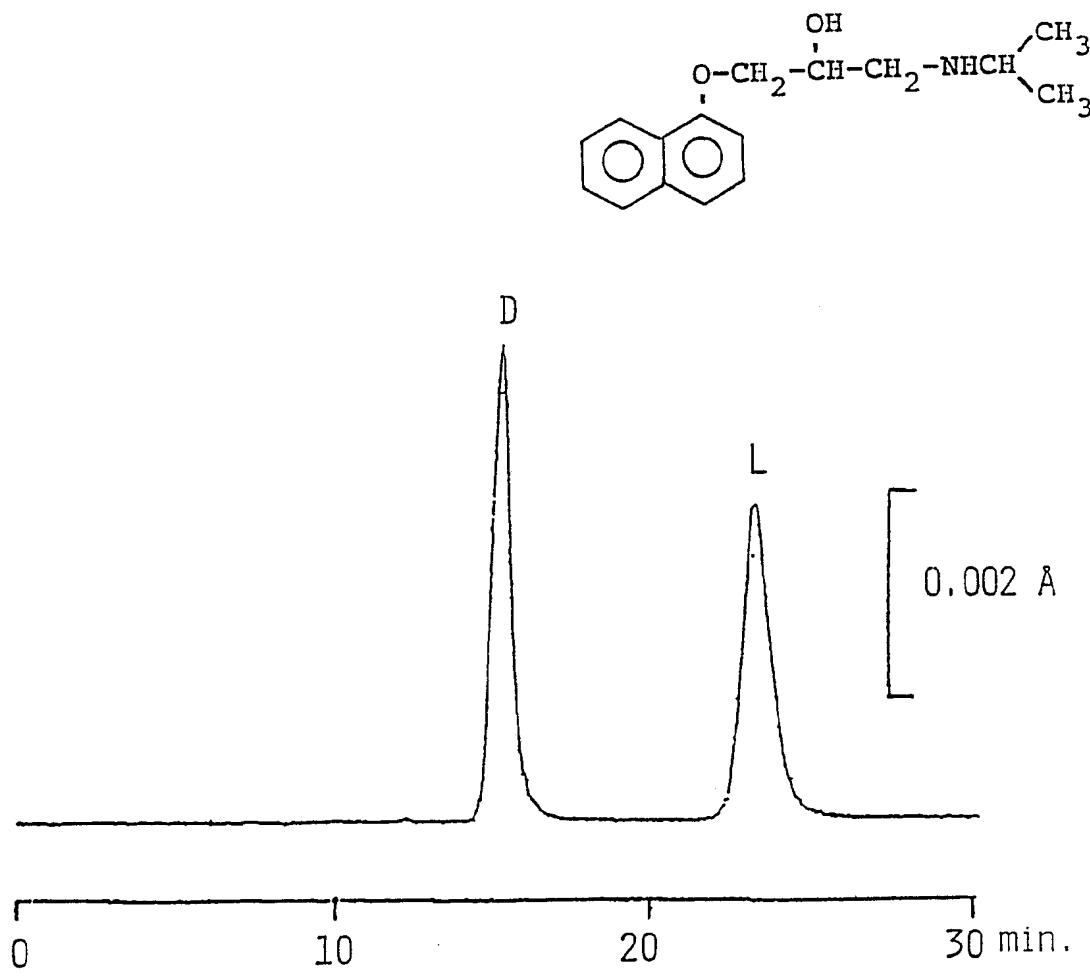
SEPARATION OF (D,L) -PROPRANOLOL

FIG. 1

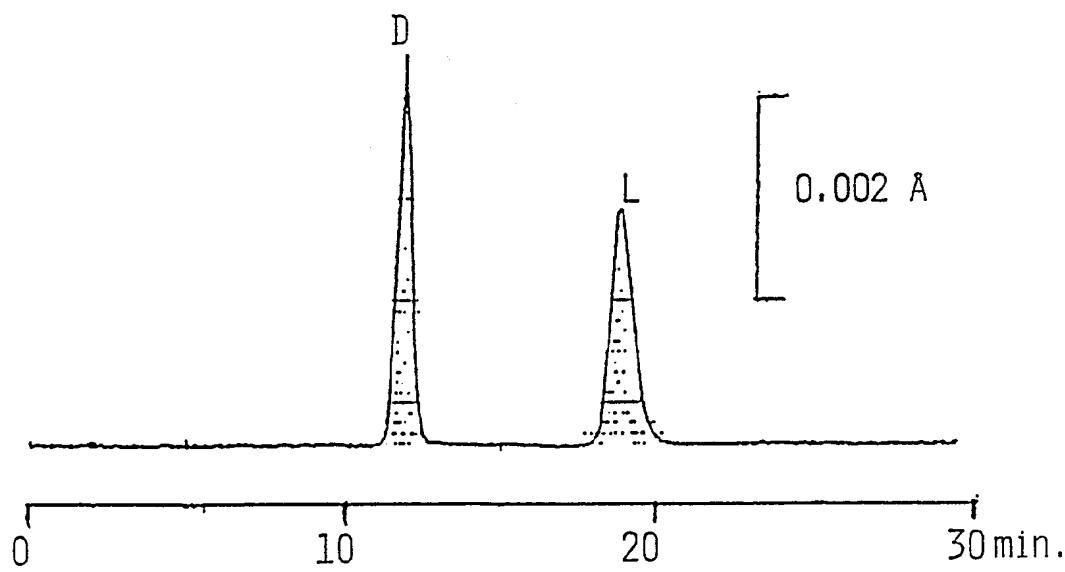
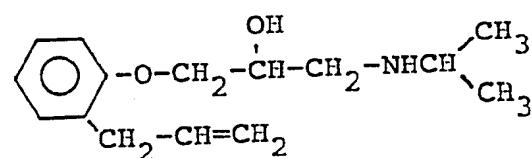
SEPARATION OF (D,L)-ALPRENOLOL

FIG. 2

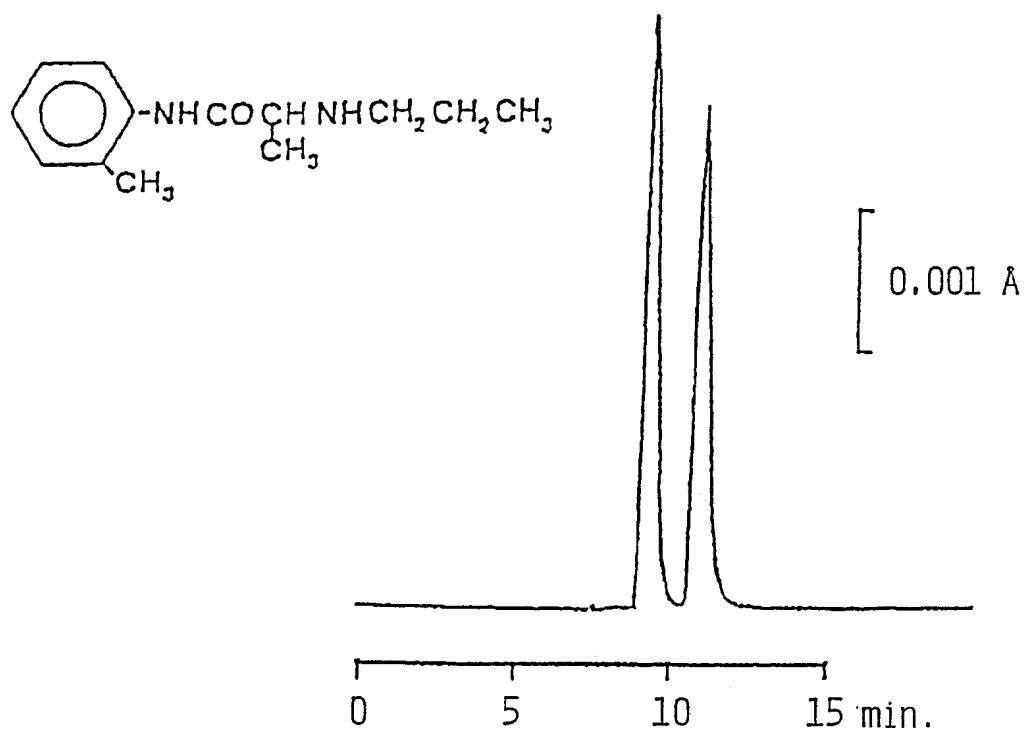
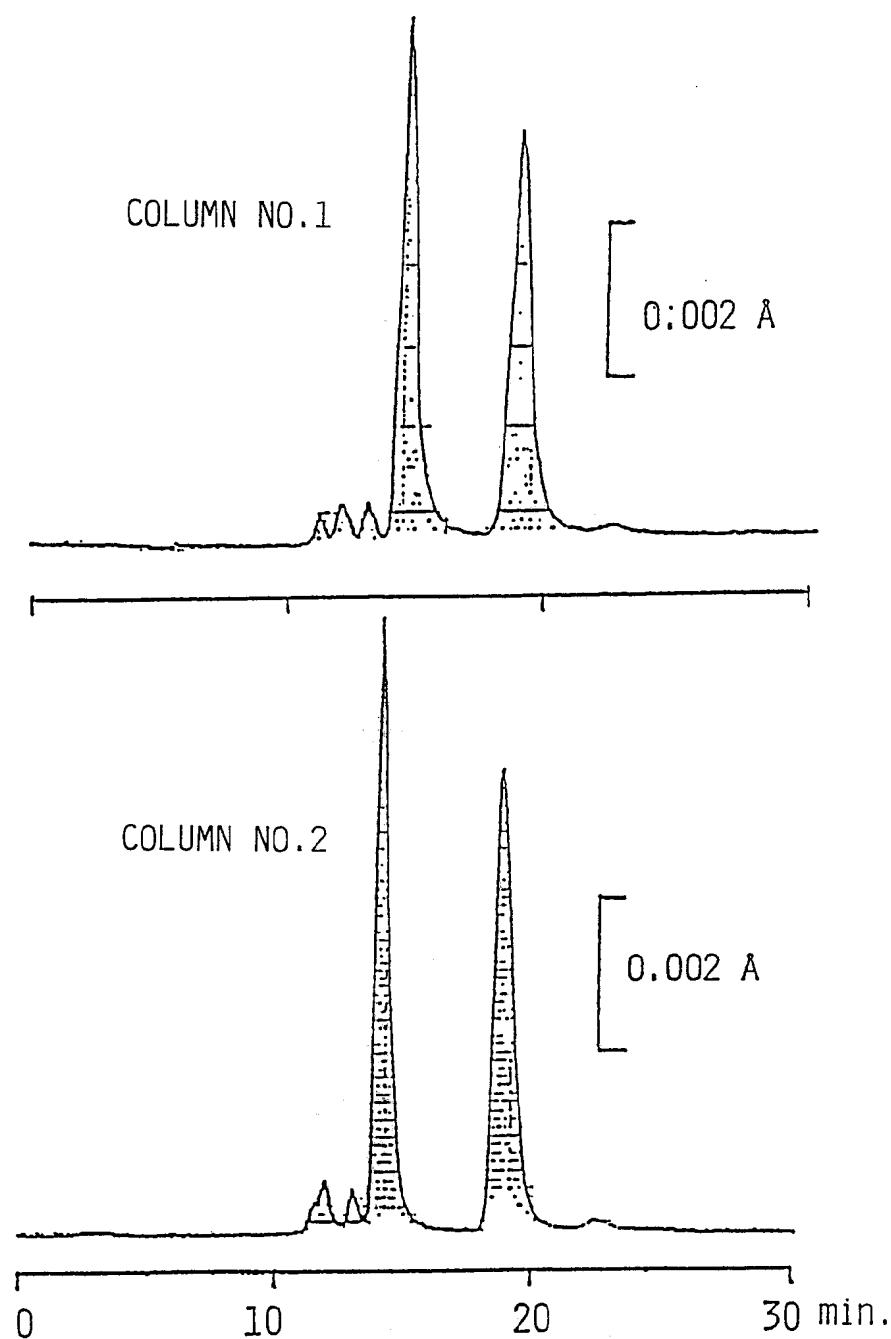
ENANTIOMER SEPARATION OF PRILOCAINE

FIG. 3

REPRODUCIBILITY OF CHROMATOGRAPHIC
CAPACITY OF COLUMNS

DISSOLVED SUBSTANCE:H 125/72 FIG.4

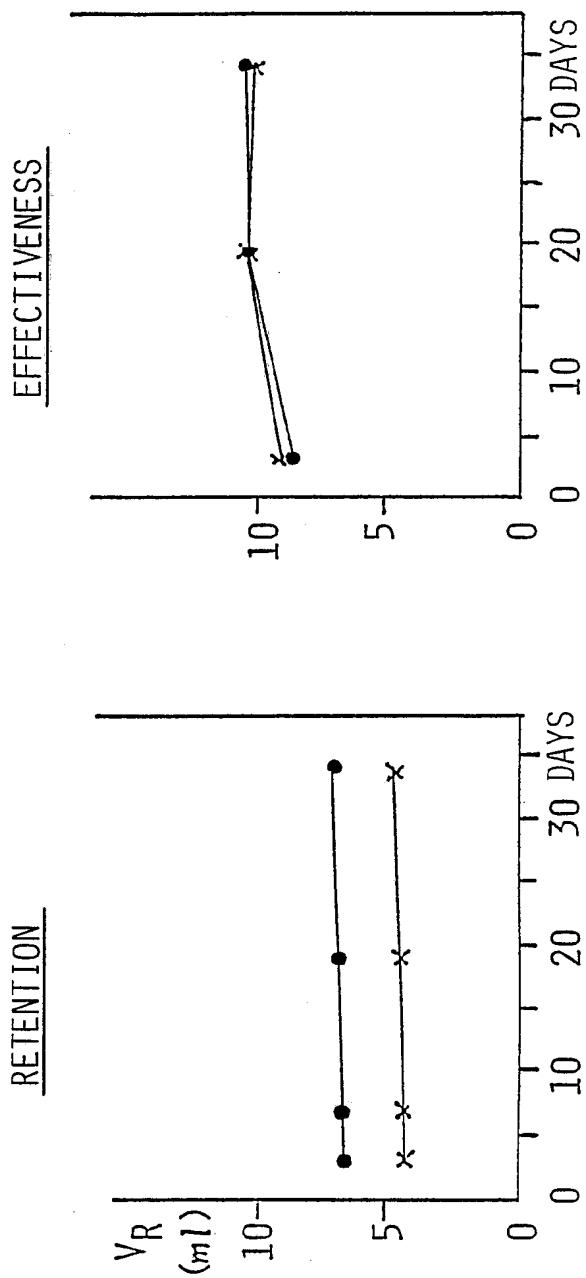


STABILITY OF CELLULASE SILICA GEL

DISSOLVED SUBSTANCE: (D,L)-PROPRANOLOL

X=D
●=L

FIG. 5



INTERNATIONAL SEARCH REPORT

International Application No PCT/SE 91/00042

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: C 07 B 57/00, C 12 P 41/00		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC5	C 07 B; C 12 P	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched ⁸		
SE,DK,FI,NO classes as above		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A	EP, A2, 0128886 (CHROMTECH AB) 19 December 1984, see the whole document --	1-9
A	Patent Abstracts of Japan, Vol 11, No 381, C464, abstract of JP 62-151196, publ 1987-07-06 NITTO ELECTRIC IND CO LTD --	1-9
P,X	Chemical Abstracts, volume 112, no. 25, 18 June 1990, (Columbus, Ohio, US), Erlandsson Per et al.: "Immobilized cellulase (CBH I) as a chiral stationary phase for direct resolution of enantiomers ", see page 546, abstract 234476t, & J. Am. Chem. Soc. 1990, 112(11), 4573-4574 --	1-9
A	Chemical Abstracts, volume 110, no. 14, 3 April 1989, (Columbus, Ohio, US), Wainer I.W. et al.: "Enzymes as HPLC Stationary phases for chiral resolutions: initial investigations with -chymotrypsin.", see, abstract 127798m, & Chromatographia 1988, 25(10), 903- 907 --	1-9
* Special categories of cited documents: ¹⁰ "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed		
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
14th May 1991	1991-05-22	
International Searching Authority	Signature of Authorized Officer	
SWEDISH PATENT OFFICE	Eva Johansson	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Chemical Abstracts, volume 111, no. 9, 28 August 1989, (Columbus, Ohio, US), Jadaud Philippe et al.: "The stereochemical resolution of enantiomeric free and derivatized amino acids using an HPLC chiral stationary phase based on immobilized -chymotrypsin: chiral separation due to solute structure or", see page 385, abstract 74096t, & Chirality 1989, 1(1), 38-44 --	1-9
A	Chemical Abstracts, volume 111, no. 22, 27 November 1989, (Columbus, Ohio, US), Jadaud Philippe et al.: "Stereochemical recognition of enantiomeric and diastereomeric dipeptides by high-performance liquid chromatography on a chiral stationary phase based upon immobilized -chymotrypsin ", see page 452, abstract 201703r, & J. Chromatogr. 1989, 476(), 165- 174 --	1-9
A	EP, A, 0164573 (BAYER AG) 18 December 1985, see the whole document -- -----	1-9

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.PCT/SE 91/00042**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on **91-03-23**
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0128886	84-12-19	DE-A- 3467511 JP-B- 2013648 JP-A- 60041619 SE-B-C- 445649 SE-A- 8303221 SE-A- 8307023 US-A- 4612121	87-12-23 90-04-04 85-03-05 86-07-07 84-12-09 84-12-09 86-09-16
EP-A- 0164573	85-12-18	DE-A- 3418374 JP-A- 60244295 US-A- 4985364	85-11-21 85-12-04 91-01-15